T/AU99/00896
09/807519

REC'D 08 DEC 1999
WIPO PCT

Patent Office
Canberra

I, KIM MARSHALL, MANAGER PATENT OPERATIONS hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 6556 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION, THE UNIVERSITY OF WESTERN SYDNEY (NEPEAN) and PIG RESEARCH AND DEVELOPMENT CORPORATION filed on 16 October 1998.

WITNESS my hand this
Thirtieth day of November 1999

KIM MARSHALL
MANAGER PATENT OPERATIONS

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

AUSTRALIA

Patents Act 1990

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

UNIVERSITY OF WESTERN SYDNEY (NEPEAN)

PIG RESEARCH AND DEVELOPMENT CORPORATION

PROVISIONAL SPECIFICATION

Invention Title:

Delivery system for porcine somatotropin

The invention is described in the following statement:

Delivery System for porcine somatotropin

FIELD OF THE INVENTION

The present invention relates to an expression construct for delivering an exogenous polypeptide to a host. The present invention also relates to recombinant cells which include this expression construct and to semi-permeable capsules which include the recombinant cells.

BACKGROUND OF THE INVENTION

In mammals, somatotropin (growth hormone) is normally secreted from the pituitary gland. However, exogenous administration of somatotropin to pigs has been shown to improve feed efficiency 15-20%, increase daily weight gain 10-15%, reduce carcass fat 10-20%, increase lean meat content 5-10% and reduce feed intake. Unfortunately, somatotropin (which is a small protein of 190 amino acids) is susceptible to gastric acids and protein digestion hence daily injections are required in order to be efficacious. Currently, welfare and ethical issues discourage the use of the pneumatic pST injection gun and the costs of daily administration restrict industry-wide adoption.

Recent advances in gene therapy have enabled the development of strategies which avoid the dependence on autologous target cells and immunosuppressive therapy by utilising transfected cells encapsulated in a semipermeable alginate-poly-L-lysine-alginate (APA) membrane. The APA capsule environment is compatible with cell viability and growth so that transfected cells remain viable, secreting growth factors, for extended periods. The APA is permeable to small proteins and consequently gene expression can be controlled by external means. The APA barrier inhibits immune surveillance and cell rejection events so that non-host, highly expressing, cells can be employed in the capsule. The APA barrier may also prevent uncontrolled proliferation of the transfected cells in the recipient host. The APA capsule can be removed, potentially reused, in order to negate the concerns regarding consumption of transgenic material. Further, if the capsule is damaged by severe tissue trauma a normal host-graft rejection would destroy the implanted cells.

SUMMARY OF THE INVENTION

The present inventors have now found that ligation of an insulin secretory signal to a heterologous gene sequence prior to introduction of the gene sequence into a host cell results in a surprising increase in the level of secretion of the heterologous gene product. This finding has led to the development of an improved gene delivery system involving encapsulation of recombinant cells for implantation into a host.

Accordingly, in a first aspect the present invention provides an expression cassette including a sequence encoding the insulin secretory signal operably linked to a heterologous sequence encoding a polypeptide.

By "heterologous sequence" we mean a sequence other than a sequence encoding insulin.

By "operably linked" we mean that the insulin secretory signal sequence is contiguous and in reading phase with the heterologous coding sequence.

The term "insulin secretory signal" refers to the secretory signal highlighted in Figure 2. It will be appreciated by those skilled in the art, however, that a number of modifications may be made to the secretory signal without deleteriously affecting the biological activity of the signal. This may be achieved by various changes, such as sulfation, phosphorylation, nitration and halogenation; or by amino acid insertions, deletions and substitutions, either conservative or non-conservative (eg. D-amino acids, desamino acids) in the peptide sequence where such changes do not deleteriously affect the overall biological activity of the secretory signal.

The heterologous sequence may encode any polypeptide, other than insulin, of interest. For example, the heterologous sequence may encode a hormone, cytokine, receptor agonist or antagonist, pheromone or enzyme. In a preferred embodiment, the heterologous sequence encodes a growth hormone. Preferably, the growth hormone is somatotropin.

In a second aspect the present invention provides a vector including an expression cassette of the first aspect. The vector may be any suitable vector for introducing the expression cassette into a cell. Suitable vectors include viral vectors and bacterial plasmids.

The expression cassette of the first aspect of the present invention, or the vector of the second aspect, may further include one or more elements which regulate gene expression. Examples of suitable regulatory elements

include the Melatonin Response Element (MRE) (as described in *Schrader et al, 1996*, the entire contents of which are incorporated herein by reference), and/or rapamycin mediated transcription factors (as described in *Magari et al, 1997*, the entire contents of which are incorporated herein by reference). In a preferred embodiment, the regulatory elements enable pulsatile expression of the polypeptide of interest.

In a third aspect the present invention provides a recombinant cell which includes an expression cassette according to the first aspect of the present invention.

The recombinant cell may be a bacterial, yeast, insect or mammalian cell. In a preferred embodiment, the recombinant cell is a mammalian cell. In a further preferred embodiment, the cell is a rat myoblast (L6) cell.

In a fourth aspect the present invention provides a method of producing a polypeptide which includes culturing a recombinant cell of the third aspect under conditions enabling the expression and secretion of the polypeptide and optionally isolating the polypeptide.

The recombinant cell(s) of the present invention may be encapsulated in a semi-permeable matrix for delivery or implantation in a host.

Accordingly, in a fifth aspect the present invention provides a capsule for implantation in a host, the capsule including a semi-permeable membrane which encapsulates one or more recombinant cells according to the third aspect of the present invention.

In a preferred embodiment, the semi-permeable membrane is an alginate-poly-L-lysine-alginate (APA) membrane. The preparation of an APA semi-permeable membrane is described in *Basic et al, 1996*, the entire contents of which are incorporated herein by reference.

In a sixth aspect the present invention provides a method of administering a polypeptide to a host which includes administering to the host an expression cassette according to the first aspect of the present invention.

In a seventh aspect the present invention provides a method of administering a polypeptide to a host which includes implanting in the host a capsule according to the fifth aspect of the present invention.

The host may be any animal or human. In a preferred embodiment, the host is a livestock animal. In a further preferred embodiment, the host is

selected from the group consisting of grazing cattle, feed-lot cattle, dairy cows, pigs, and poultry.

It will be appreciated by those skilled in the art that the present invention provides an improved system for the delivery of genetic material to a host. The ligation of the insulin secretory signal to a biologically active polypeptide leads to increased secretion of the polypeptide from recombinant cells. Following secretion, the secretory signal may be cleaved leaving the biologically active polypeptide. The recombinant cells, when encapsulated in a semipermeable matrix, have the capacity to secrete significant amounts of the biologically active polypeptide and the semi-permeable membrane enables control of gene expression by external means. Implantation of the encapsulated recombinant cells provide an advantage in that the implantation requires minimal surgery. Further, the semi-permeable matrix reduces immune surveillance and cell rejection which means that non-host cells can be employed in the capsule.

In a preferred embodiment, the semi-permeable membrane is durable which provides an advantage in that it may limit cell growth thereby preventing uncontrolled proliferation in the recipient host. The capsules provide a further advantage in that they may be removed and reused.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting Examples and Figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Insulin secretory signal - pST gene construct

Figure 2: Insulin secretory signal - pST peptide sequence

Figure 3: Rate of weight gain (from day 0) for control and individual pST-L6IXS treated pigs

Figure 4: Percentage weight gain for control and individual pST-L6IXS treated animals

Figure 5: Plasma, pST levels for control and individual pST-L6IXS treated animals

Figure 6: Ex-vivo assessment of secretion of pST from capsules for a 24 hr period following removal from host animal

Figure 7: Plate 1- Appraisal of pST-L6IXS capsule administration site
Plate 2 - Placement of pST-L6IXS capsule in culture media for ex-vivo assessment

DETAILED DESCRIPTION OF THE INVENTION

Example 1: Cloning of the ISS-pST construct

The pST gene was obtained from Southern Cross Biotechnology in an *E. coli* bacterium. The plasmid containing the pST gene, pMG939, was isolated from the bacterium using standard plasmid preparation techniques. The PCR primers were designed to amplify the pST gene, add an *Xho* I site to the 5' end and an *Xba* I site to the 3' end to enable ligation events.

The modified pST gene sequence was subsequently ligated to a secretory signal sequence (ISS) derived from the preproinsulin cDNA. *Nhe* I (GCTAGC) and *Xba* I (TCTAGA) restriction sites were constructed in front of the ISS start codon and after the 3' terminal codon of pST, respectively, to allow incorporation into the pCI-neo plasmid (Promega). The pST fusion construct was subsequently isolated and sequenced to verify the coding region (Figure 1).

Transfection of rat myoblast (L6) cells (pST gene incorporation into cells) was performed, with LipoTAXI (Stratagene), 2hrs after the L6 cells were trypsin treated. pST transfected L6 cell clones were maintained in culture, selected with G418, until $>10^7$ cells were generated. Aliquots (2ml) of the culture supernatant were stored at -20°C prior to assessment of pST concentrations in a pST radioimmunoassay (RIA) established by Dr P. Wynn at Sydney University (Camden). The RIA sensitivity was deemed to be >0.4ng/ml with CV's in the order of 12.4%. The polyclonal antisera was raised in guinea pigs with a pST peptide antigen. The RIA results (Table 1) indicate that the pST gene construct produced protein (Figure 2) which is recognised by polyclonal antisera raised against the native form of pST, purified from porcine pituitary glands. L6 Clones pCI/pst-1..5 were generated from the modified transfection technique as described below.

Modified transfection protocol

Characteristically, L6 cells adhere to culture plates and require detachment with trypsin to passage cells; transfection is routinely performed 24hrs later. This procedure resulted in L6 cell clones (n=10) secreting pST at 6-18 ng/ml. Applying LipoTAXI (Promega) and the ISS/pST plasmid to the

L6 cells 2hrs after trypsin treatment increased the secretion rate of pST 10-20 fold (>180ng/ml, n=5 clones). This higher pST secretion rates reduce the number of cells (capsules) required to enhance growth.

TABLE 1: Concentrations (ng/ml) for each clone transfected with ISS-pST.

L6 clone	pST (ng/ml)
pCI/pst-1*	182
pCI/pst-2*	188
pCI/pst-3*	188
pCI/pst-4*	140
pCI/pst-5*	200
pCI/pst-6	17
pCI/pst-7	12
pCI/pst-8	8
pCI/pst-9	9
pCI/pst-10	7
pCI/pst-11	7
pCI/pst-12	10
pCI/pst-13	8
pCI/pst-14	6
pCI/pst-15	18

Example 2: Preparation of the porcine somatotropin-rat myoblast (L6) immunoneutral expression system (pST-L6IXS)

The encapsulation procedure described in *Basic et al, 1996*, was followed with the following modifications.

Encapsulation of cells at room temperature, utilises calcium chloride (or lactate) [100mM] to gel the alginate [1.5% w/v] droplets followed immediately by washing with saline (0.9% NaCl) then resuspending in poly-L-lysine [0.05%] for 5 min. Calcium chloride crosslinking for 10min at 37°C resulted in an alginate matrix that was more compatible with cell viablity.

After the poly-L-lysine coating and saline washes another alginate layer is added. Sodium citrate [55mM] treatment for 4min at room temperature softens the capsule to a consistency that increases the difficulty of further manipulation. Cell viablity is apparently reduced to <35% with 4 min exposure to sodium citrate. Placing the capsules in a cell strainer prior

to sodium citrate treatment enabled 1min exposure, at 37°C, improving cell viability to >98%.

Procedural and equipment modifications to the encapsulation protocol improved the efficiency (time and resources) of encapsulation with routine increases in cell viability in the order of 64%.

Example 3: Pilot experiment involving implantation of pST-L6IXSin pigs

Preliminary results obtained with the pST-L6IXS, administered to growing mice, indicate enhanced growth characteristics. In a pilot experiment with male pigs (n=9, mean live weight 61 kg) varying numbers of pST-L6IXS were administered in different sites (3 capsules, i.m. in the neck muscle, 3 capsules s.c. in the neck, 10 capsules s.c. at the base of the ear, 20 capsules i.m. in the neck or 29 capsules i.m. in the neck of individual animals on day 0. Blood samples (10ml) were collected via jugular venipuncture and P2 ultra-sound (us) measurements were recorded at -14, 0, 7, 14, 21, 28 and 36 days post administration. The sites of pST-L6IXS administration were monitored for tissue reaction events throughout the experiment. On day 36 animals were euthanased and carcass analysis (back fat depth, BF(mm); eyemuscle area, EMA(cm); forearm bone length, BONE(cm); heart weight, HEART(gm); spleen weight, SPLEEN(gm) and liver weight, LIVER(gm) were recorded (see Table 2) and pST-L6IXS recovered. Figure 3 represents the rate of gain (from day 0) for control (con, mean±SE, n=4) and individual values for pST-L6IXS treated pigs. Percentage weight gain, over the pST-L6IXS treatment is presented in Figure 4 with the mean±SE for control (con) pigs and individual pST-L6IXS treated animals. Plasma pST (ng/ml) was determined by radioimmunoassay (RIA) and presented in Figure 5, with mean±SE control (con) and individual concentrations for pST-L6IXS treated pigs. At slaughter the site of pST-L6IXS capsule administration was appraised (Figure 7, Plate 1, arrow) prior to removal and placement in culture media for ex-vivo assessment (Figure 7, Plate 2) of 24 hour secretion of pST (Figure 6). No apparent tissue damage or immune reactions were observed either i.m. or s.c. at day 36. However, the capsules placed in the ear (s.c.) appeared to be highly vascularised and were 100% recoverable. The capsules placed in the neck region were <10% recoverable.

Table 2

pST-L6IXS PILOT EXPERIMENT:
Pigs (male) supplied byWestmill piggery (Young, NSW)
Experiment at EMAI, maximum security piggery.
ACEC Ref No: 98/20

				LIVEWEIGHT(kg)													
				Date													
				###	##	###	##	###	###	9/07/98							
				Day						(slaughter)	CARCASS						
	Pen	Treatment	Animal	-14	0	7	14	21	28	36	P2us(mm)	BF(mm)	EMA(cm)	BONE(cm)	HEART(gm)	SPLEEN(gm)	LIVER(gm)
	A	con	291	24	67	NR	NR	89	95	100	11	9	54.5	24.5	388.6	159.8	1720.2
C	A	con	292	25	61	NR	NR	84	90	90	8	10	54.9	23.7	381.5	103.2	1703.6
C	B	con	294	22	74	NR	NR	94	103	104	12	15	46.5	24.4	391.5	173.2	1636.5
C	B	con	295	22	55	NR	NR	76	84	91	9	7	50.6	20.0	396.6	138.2	1561.8
T	B	3sc neck*	297	23	67	NR	NR	85	90	91	9	12	45.2	23.5	385.3	177.0	1817.7
		* infected capsule site								CvTp<0.05	nsd	nsd	CvTp<0.06	nsd	CvTp<0.05	nsd	nsd

The pST-L6IXS remained patent over 36 days *in vivo* and appeared to proliferate within the capsule (Plate 2) which can be removed in order to negate the concerns regarding consumption of transgenic material. Further, if the capsule is damaged (i.e. by severe tissue trauma) a normal host-graft rejection destroys the L6 cells preventing propagation of transfected material. Experiments in mice and pigs have demonstrated that pST-L6IXS are efficacious in altering plasma pST, enhancing growth characteristics and potentially immune competence of animals.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this sixteenth day of October 1998

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION
UNIVERSITY OF WESTERN SYDNEY (NEPEAN)
PIG RESEARCH AND DEVELOPMENT CORPORATION
Patent Attorneys for the Applicant:

F B RICE & CO

References

Basic D., et al, (1996) Microencapsulation and transplantation of genetically engineered cells: A new approach to somatic gene therapy. Art. Cells, Blood subs. and Immob. Biotech 24(3): 219-255.

Magari et al, (1997) Pharmacological control of humanised gene therapy system implanted into nude mice. J. Clin. Invest. 100: 2865-2872

Schrader et al, (1996) Identification of natural monomeric response elements of the nuclear receptor R2R/ROR. They also bind to COUP-TF homodimers. J. Biol. Chem. 271:19732-19736

FIGURE 1: ISS-pST gene construct

1 **GCTAGC**ATGG CCCTGTGGAT GCGCCTCCTG CCCCTGCTGG CGCTGCTGGC

51 CCTCTGGGGA CCTGACCCAG CCGCAGCC**CT CGAG**ATGTTT CCAGCTATGC

101 CACTTTCTTC TCTGTTCGCT AACGCTGTTC TTCGGGCCCA GCACCTGCAC

151 CAACTGGCTG CCGACACCTA CAAGGAGTTT GAGCGCGCCT ACATCCCGGA

201 GGGACAGAGG TACTCCATCC AGAACGCCCA GGCTGCCTTC TGCTTCTCGG

251 AGACCATCCC GGCCCCCACG GGCAAGGACG AGGCCCAGCA GAGATCGGAC

301 GTGGAGCTGC TGCGCTTCTC GCTGCTGCTC ATCCAGTCGT GGCTCGGGCC

351 CGTGCAGTTC CTCAGCAGGG TCTTCACCAA CAGCCTGGTG TTTGGCACCT

401 CAGACCGCGT CTACGAGAAG CTGAAGGACC TGGAGGAGGG CATCCAGGCC

451 CTGATGCGGG AGCTGGAGGA TGGCAGCCCC CGGGCAGGAC AGATCCTCAA

501 GCAAACCTAC GACAAATTTG ACACAAACTT GCGCAGTGAT GACGCGCTGC

551 TTAAGAACTA CGGGCTGCTC TCCTGCTTCA AGAAGGACCT GCACAAGGCT

601 GAGACATACC TGCGGGTCAT GAAGTGTCGC CGCTTCGTGG AGAGCAGCTG

651 TGCCTTCTAG **TCTAGA**

ATG...GCC- insulin secretory signal.

GCTAGC- Nhe I restriction site incorporated into construct in order to ligate into plasmid.

CTCGAG- Xho I restriction site incorporated into construct in order to ligate secretory signal and pST.

TCTAGA- Xba I restriction site incorporated into construct in order to ligate into plasmid.

FIGURE 2: ISS-pST peptide sequence.

1 MALWMRLLPL LALLALWGPD PAAALEMFPA MPLSSLFANA VLRAQHLHQL

51 AADTYKEFER AYIPEGQRYS IQNAQAAFCF SETIPAPTGK DEAQQRSDVE

101 LLRFSLLLIQ SWLGPVQFLS RVFTNSLVFG TSDRVYEKLK DLEEGIQALM

151 RELEDGSPRA GQILKQTYDK FDTNLRSDDA LLKNYGLLSC FKKDLHKAET

201 YLRVMKCRRF VESSCAF

MAL....AAA- insulin secretory signal, cleaved upon sectretion of pST.

LE- function of XhoI cleavage site; result in no predicted secondary structural changes to pST.

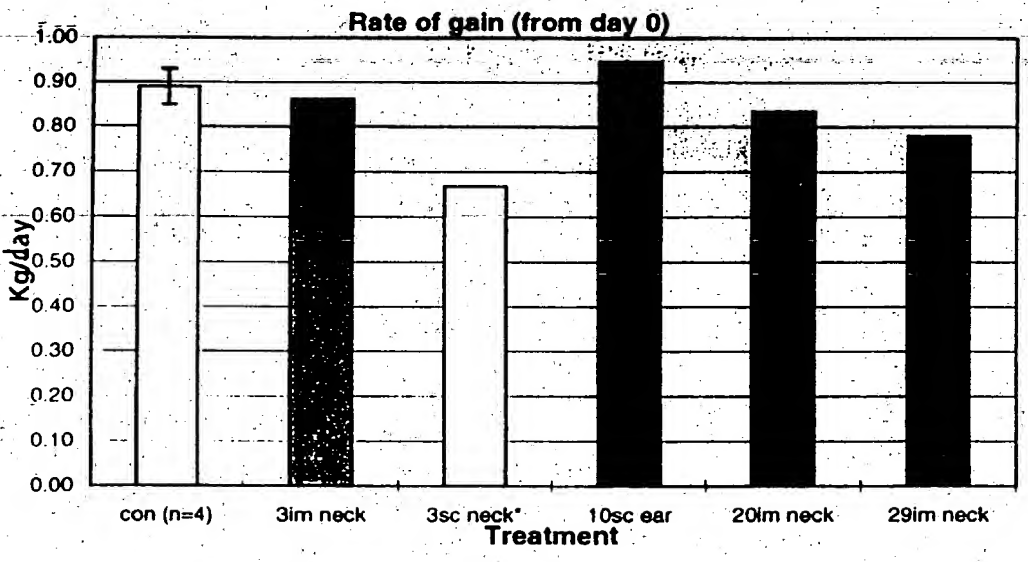
Rate of gain (from day 0)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Kg/day
con (n=4)
3im neck
3sc neck*
10sc ear
20im neck
29im neck
Treatment

Figure 3

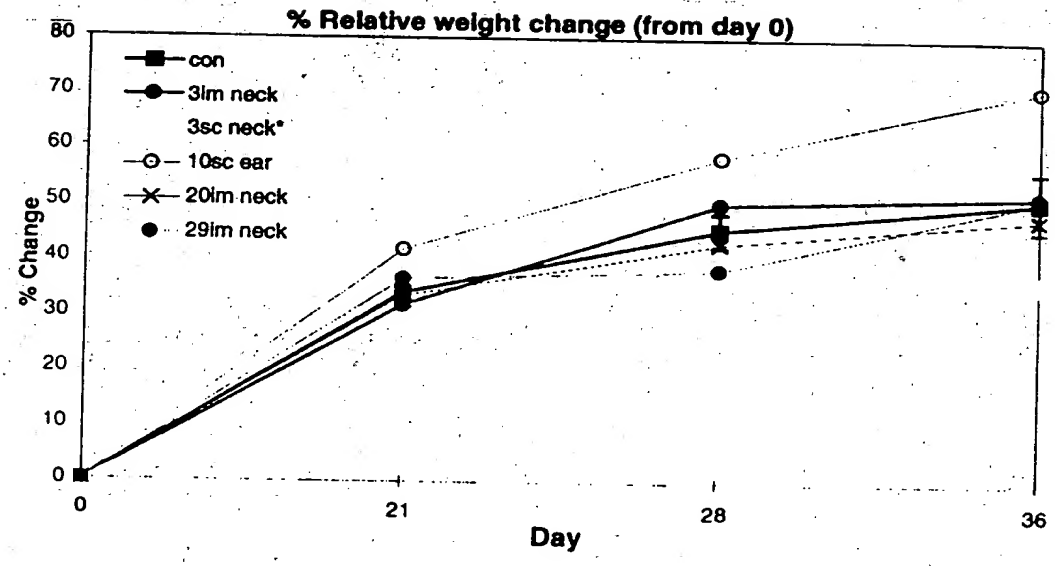
% Relative weight change (from day 0)
con
3im neck
3sc neck*
10sc ear
20im neck
29im neck
% Change
80
70
60
50
40
30
20
10
0
0
21
28
36
Day

Figure 4

Figure 5

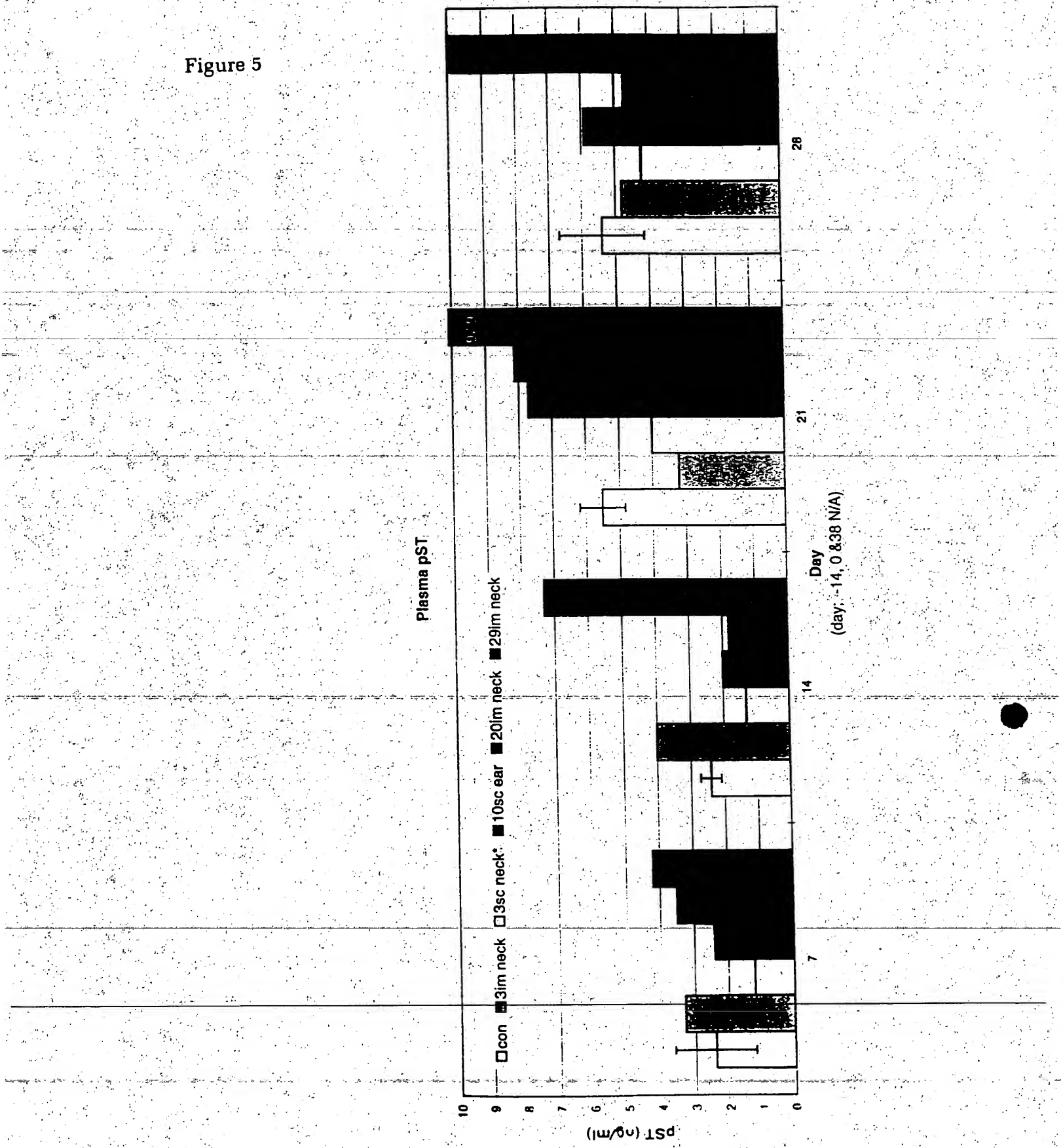
Plasma pST
pST (ng/ml)
10
9
8
7
6
5
4
3
2
1
0
7
14
21
28
Day
(day; -14, 0 &38 N/A)
con
3im neck
3sc neck
10sc ear
20im neck
29im neck

Figure 6

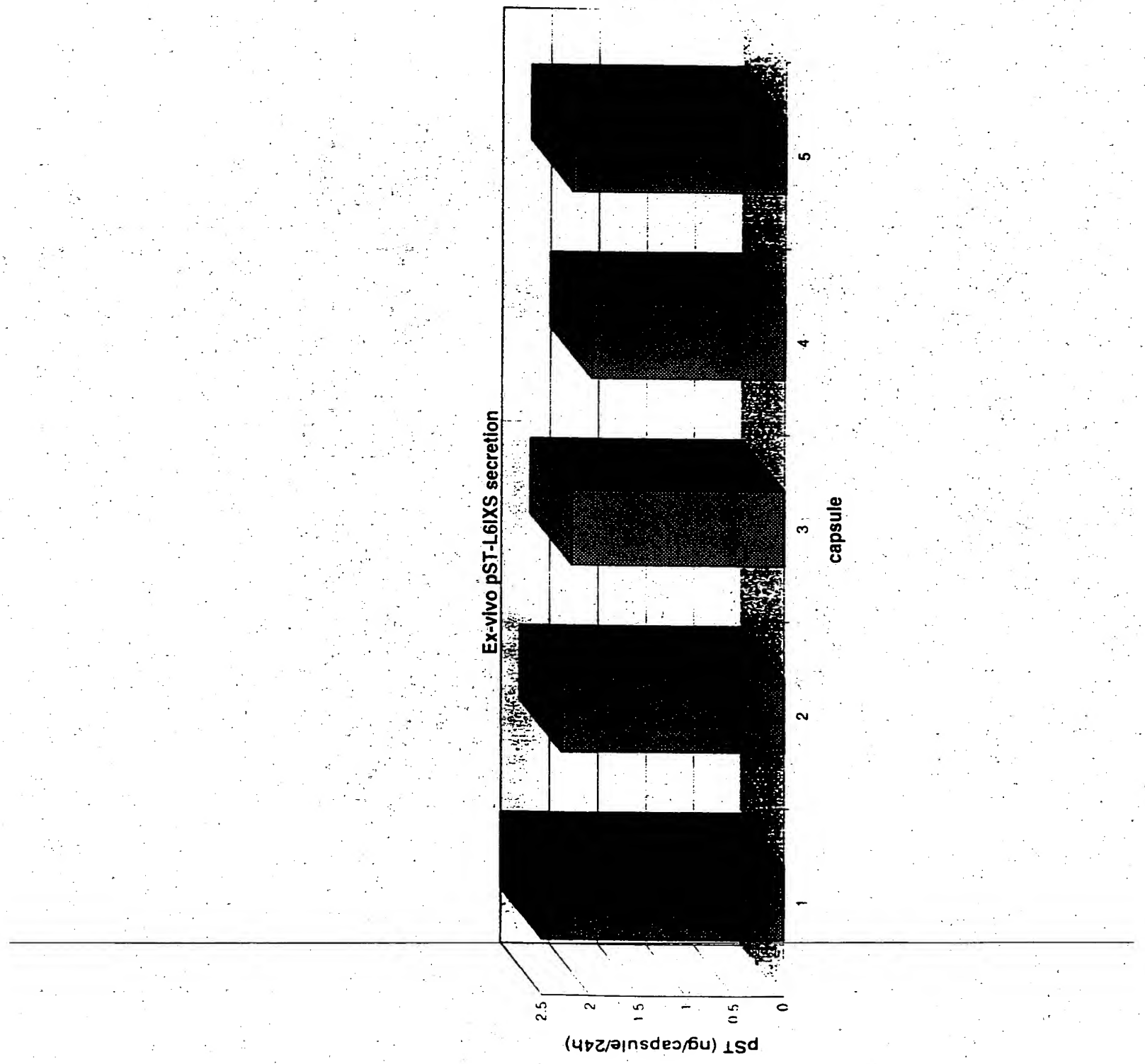
Ex-vivo pST-L6IXS secretion
pST (ng/capsule/24h)
0
0.5
1
1.5
2
2.5
capsule
1
2
3
4
5

Plate 1

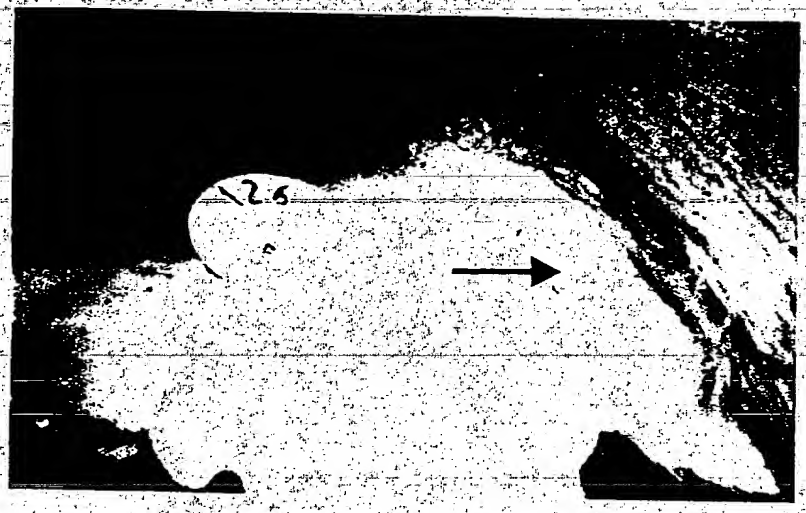

Plate 2

Figure 7